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Published in:
Journal of Bacteriology

Publication date:
1991

JOURNAL OF BACTERIOLOGY, Oct. 1991, p. 6030–6037
0021-9193/91/196030-08$02.00/0

Vol. 173, No. 19

Malolactic Fermentation: Electrogenic Malate Uptake and Malate/Lactate Antiport Generate Metabolic Energy

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Received 1 May 1991/Accepted 24 July 1991

The mechanism of metabolic energy production by malolactic fermentation in *Lactococcus lactis* has been investigated. In the presence of L-malate, a proton motive force composed of a membrane potential and pH gradient is generated which has about the same magnitude as the proton motive force generated by the metabolism of a glycolytic substrate. Malolactic fermentation results in the synthesis of ATP which is inhibited by the ionophore nigericin and the F_0F_1-ATPase inhibitor *N,N*-dicyclohexylcarbodiimide. Since substrate-level phosphorylation does not occur during malolactic fermentation, the generation of metabolic energy must originate from the uptake of L-malate and/or excretion of L-lactate. The initiation of malolactic fermentation is stimulated by the presence of L-lactate intracellularly, suggesting that L-malate is exchanged for L-lactate. Direct evidence for heterologous L-malate/L-lactate (and homologous L-malate/L-malate) antiport has been obtained with membrane vesicles of an *L. lactis* mutant deficient in malolactic enzyme. In membrane vesicles fused with liposomes, L-malate efflux and L-malate/L-lactate antiport are stimulated by a membrane potential (inside negative), indicating that net negative charge is moved to the outside in the efflux and antiport reaction. In membrane vesicles fused with liposomes in which cytochrome *c* oxidase was incorporated as a proton motive force-generating mechanism, transport of L-malate can be driven by a pH gradient alone, i.e., in the absence of L-lactate as countersubstrate. A membrane potential (inside negative) inhibits uptake of L-malate, indicating that L-malate is transported as an electronegative monoanionic species (or dianionic species together with a proton). The experiments described suggest that the generation of metabolic energy during malolactic fermentation arises from electrogenic malate/lactate antiport and electrogenic malate uptake (in combination with outward diffusion of lactic acid), together with proton consumption as a result of decarboxylation of L-malate. The net energy gain would be equivalent to one proton translocated from the inside to the outside per L-malate metabolized.

Malolactic fermentation is carried out by species of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* (2, 17, 22). In this pathway, L-malate enters the cells and is decarboxylated by malolactic enzyme to yield L-lactate and carbon dioxide, after which L-lactate and carbon dioxide leave the cell. Although the decarboxylation of L-malate is a non-energy-yielding reaction catalyzed by a single enzyme, malolactic fermentation supplies the cell with additional metabolic energy (17, 22). It has been proposed that electrogenic efflux of L-lactate and/or carbon dioxide is responsible for the metabolic energy produced (2). Since the decarboxylation of L-malate by the lactic acid bacteria is analogous to the decarboxylation of oxalate by *Oxalobacter formigenes* (1), it has been suggested that the metabolic energy may be gained from electrogenic malate/lactate antiport analogous to the energy generation by oxalate/formate antiport (18).

Since substrate-level phosphorylation or direct ion extrusion by a membrane-bound decarboxylase (3) does not occur during malolactic fermentation, the generation of metabolic energy must originate from the movement of L-malate, L-lactate, and/or carbon dioxide across the membrane. Additionally, the cell could take advantage of the fact that a proton is consumed during the intracellular decarboxylation of L-malate. Assuming that carbon dioxide diffuses out of the cell without affecting the pH gradient, three distinct mechanisms of metabolic energy generation during malolactic fermentation can be operative: electrogenic malate/lactate antiport, electrogenic malate uptake, and electrogenic lactate efflux (Fig. 1). In line with the low pH at which malolactic fermentation is operative (22), transport of monoanionic malate is assumed. For each of the proposed mechanisms, the overall transport process is electrogenic; i.e., a membrane potential is generated either by the antiport reaction, malate uptake, or lactate efflux, and a pH gradient is generated as a result of proton consumption in the cytoplasm (Fig. 1). In the three mechanisms shown, the linkage of the transport processes to the decarboxylation of L-malate will result in the equivalent of one proton translocated per L-malate molecule metabolized.

To discriminate between the three mechanisms of energy generation (Fig. 1), transport experiments were conducted in which the effects of membrane potential, pH gradient, and countersubstrate(s) on the uptake and efflux of L-malate were analyzed. For practical reasons (membranes are less permeable for L-malate than for L-lactate), malate/lactate antiport was assayed in the direction opposite the in vivo reaction. Although malolactic fermentation is commonly studied in *Leuconostoc oenos* or *Lactobacillus plantarum*, *Lactococcus lactis* was chosen for these studies since appropriate mutants were available or could be isolated relatively easily. We present evidence for a secondary transport system that catalyzes electrogenic malate/lactate antiport as

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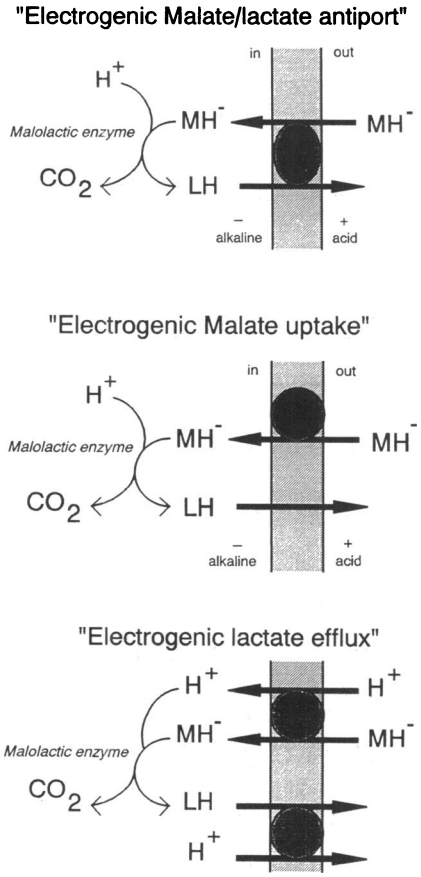

FIG. 1. Possible mechanisms for the generation of metabolic energy by malolactic fermentation based on electrogenic malate/lactate antiport, electrogenic malate uptake, and electrogenic lactate efflux. MH^-, monoanionic L-malate; LH, L-lactic acid.

well as electrogenic malate uptake in *L. lactis* IL1403. This system forms the basis for the generation of metabolic energy during malolactic fermentation.

MATERIALS AND METHODS

Strains and culture conditions. *L. lactis* IL1403 (wild-type, plasmid-free strain), IL1441 (streptomycin-resistant derivative of IL1403), and isogenic mutants defective in L-malate transport (UV1) have been described elsewhere (23). Spontaneous mutants defective in malolactic enzyme (SO1, SO2, and SO5) were isolated from the streptomycin-resistant isogenic strain IL1441 on E modified medium (23). *L. lactis* ML3 served as a control organism deficient in malolactic fermentation. Cells were grown at 30°C in complex medium (MRS) supplemented with glucose or galactose (25 mM) and with or without potassium-L-malate (50 mM) (21).

Isolation of membrane vesicles. Membrane vesicles of *L. lactis* IL1403 (wild type), IL1441-SO1 (malolactic enzyme-deficient mutant), and ML3 were prepared by osmotic lysis as described previously (16). Cells were grown in MRS supplemented with glucose plus potassium-L-malate.

Fusion of liposomes and proteoliposomes with membrane vesicles. Cytochrome *c* oxidase, isolated from beef heart mitochondria (24), was reconstituted into liposomes containing acetone-ether-washed *Escherichia coli* phospholipids by dialysis as described previously (4). *L. lactis* membrane vesicles (250 μl; 2.0 mg of protein) and cytochrome *c* oxidase proteoliposomes (1 ml; 20 mg of phospholipid; 2.25 nmol of cytochrome *c* oxidase) were mixed and fused by freeze-thaw sonication (8 s at an amplitude of 4 μm) as described previously (4, 5), resulting in hybrid membranes. By the same procedure, membrane vesicles were fused with liposomes devoid of cytochrome *c* oxidase.

Transport assays. (i) Intact cells. Cells were harvested by centrifugation, washed, and resuspended in 50 mM potassium phosphate supplemented with 2 mM $MgSO_4$, pH 5.0 (50 KPi buffer). For the transport experiments, concentrated cell suspensions were diluted to a final protein concentration of 0.5 to 1.0 mg/ml into 50 KPi buffer containing 10 mM glucose. Following 2 min of preenergization at 30°C, radioactive solutes were added; at the indicated time intervals, the uptake reaction was stopped by the addition of 2 ml of ice-cold 0.1 M LiCl. The samples were filtered over 0.45-μm-pore-size cellulose-nitrate filters (Millipore Corp.) and washed once more with 2 ml of ice-cold 0.1 M LiCl (21).

(ii) Membrane vesicles and fused membranes. For efflux and exchange experiments, membrane vesicles or membranes fused with liposomes in 50 mM potassium phosphate–0.1 M KCl supplemented with 2 mM $MgSO_4$ (KPi/KCl buffer of the indicated pH) were loaded with the appropriate concentration of radiolabelled substrates for 1 to 2 h at room temperature. The membrane vesicles or fused membranes were concentrated by centrifugation and diluted 80-fold into buffer with and without countersubstrate. Specific reaction conditions are indicated in the text or figure legends. The transport reactions were stopped at different time intervals as indicated above. In membrane vesicles fused with cytochrome *c* oxidase-containing proteoliposomes, hybrid membranes were incubated in KPi/KCl buffer of the indicated pH containing 200 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), 20 μM cytochrome *c*, and 10 mM potassium ascorbate unless indicated otherwise. After 1 min of incubation in the presence of oxygen (continuous aeration), the radiolabelled substrates were added, and uptake was assayed as described above.

Malolactic fermentation activity. Cells were washed and resuspended in 5 mM K-MES (potassium-morpholineethanesulfonic acid)–50 mM KCl–2 mM $MgSO_4$ (K-MES/KCl buffer), pH 5 (unless indicated otherwise). L-Lactate, D-lactate, acetate, benzoate, and bicarbonate (up to 100 mM, potassium salts) were added or no further additions were made, and the cells were incubated for 1 h at 30°C. Subsequently, the cells were centrifuged and resuspended to a final protein concentration of 20 to 50 mg/ml and stored on ice until use. Malolactic fermentation was started by adding 10 μl of cell suspension into 4 ml of K-MES/KCl buffer containing different concentrations of L-malate (potassium salt). Alkalinization of the medium was recorded in a buffer range in which the change in external pH was less than 0.1 pH unit and linear in time. Changes in pH were converted into nanomoles of OH^- by calibration of the cell suspension with 5- to 10-μl portions of 50 mM KOH. The measurements were performed at 30°C. Malolactic enzyme activity was measured in the same manner after permeabilization of the cells with 0.03% Triton X-100.

Measurements of membrane potential, pH, and lactate gradient. The membrane potential in *L. lactis* IL1403 cells was measured with an ion-selective tetraphenylphosphonium ion (TPP^+) electrode as described elsewhere (21). The membrane potential was calculated by using the Nernst equation from the distribution of TPP^+ between the bulk phase of the medium and the cytoplasm after correction for

concentration-dependent binding of TPP^+ to the cytoplasmic membrane (11). The estimated binding constant for TPP^+ was 30. The pH and lactate gradient in *L. lactis* IL1403 cells were estimated from the distribution of [U-^{14}C]benzoic acid and L-[U-^{14}C]lactic acid, respectively, using the silicon oil centrifugation method (21). Conditions for these measurements were similar to those of the transport experiments and malolactic fermentation except that measurements of the pH and lactate gradient were performed at 20°C.

Measurement of cytoplasmic pH with a fluorescent indicator probe. Cells were loaded with the fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5(and -6)-carboxyfluorescein (BCECF) as described elsewhere (14). Subsequently, cells were washed three times and resuspended in 50 mM K-MES buffer, pH 5.0. Loading of the cells with L-lactate was performed as described above. Fluorescence measurements with BCECF-loaded cells were performed in a cuvette containing 3 ml of 50 mM K-MES buffer of the desired pH to which 10 μl of cells (approximately 5 mg of protein per ml) were added. The suspension was stirred and thermostated at 30°C. The excitation and emission monochromator wavelengths were 502 and 525 nm with slid widths of 5 and 15 nm, respectively. The fluorescence signal was averaged over time intervals of 1 s. Calibration of BCECF fluorescence was performed in nonenergized cells after dissipation of the pH gradient by the ionophore nigericin (in combination with valinomycin), i.e., under conditions of equal cytoplasmic and external pH. The total amount of BCECF was determined at the end of each experiment by adjusting the pH to 10 to 11 with KOH (maximal fluorescence) and permeabilizing the cells with 0.1% Triton X-100.

Miscellaneous. Extraction procedures for ATP analysis and measurement of the ATP concentrations with the firefly luciferase assay have been described previously (15). Protein was measured by the method of Lowry et al. (12) with bovine serum albumin as a standard. L-Lactate was determined gas chromatographically as described elsewhere (8). For *L. lactis* cells, membrane vesicles, and fused membranes, specific internal volumes of 2.9, 4.3, and 8 μl/mg of protein were used (21).

Materials. L-[U-^{14}C]malate (51 mCi/mmol), L-[U-^{14}C]leucine (348 mCi/mmol), [*carboxyl*-^{14}C]benzoic acid (50 mCi/mmol), and L-[U-^{14}C]lactate (179.5 mCi/mmol) were obtained from Amersham (Buckinghamshire, England). All other chemical were reagent grade and were obtained from commercial sources.

RESULTS

Expression of malolactic fermentation. The malolactic fermentation activities (assayed with 5 mM L-malate) of *L. lactis* IL1403 cells grown in media supplemented with glucose, glucose plus L-malate, galactose, and galactose plus L-malate were 0.074, 0.632, 0.360, and 1.0 μmol of OH^- per min per mg of protein, respectively. Although malolactic fermentation activities were highest in cells grown on galactose plus L-malate, the cells grew poorly in the presence of galactose as the carbon source, most probably because of the absence of the plasmid-encoded tagatose phosphate pathway for galactose utilization. Therefore, experiments were performed with cells grown on MRS supplemented with glucose plus L-malate unless indicated otherwise.

Generation of metabolic energy. To demonstrate the generation of a proton motive force by malolactic fermentation, resting cells of *L. lactis* IL1403 were incubated with L-malate and the magnitudes of the membrane potential and pH gradient were determined after 5 min of metabolism (Fig. 2). The pH gradient generated by malolactic fermentation was somewhat lower than the pH gradient generated by glycolysis; the opposite was true for the membrane potential. Another noticeable difference between the generation of a membrane potential by malolactic fermentation and glycolysis was the depolarization of the membrane potential after a few minutes with glucose as the substrate, whereas the steady-state value was reached within 1 min with L-malate as the substrate (data not shown).

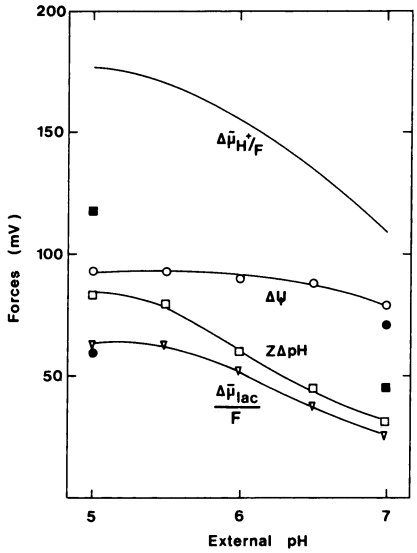

FIG. 2. Effect of external pH on the components of the proton motive force and the lactate gradient generated by malolactic fermentation and glycolysis. *L. lactis* IL1403 cells, loaded with 100 mM L-lactate, were suspended in 50 mM K-MES–50 mM potassium piperazine-N,N'-bis(2-ethanesulfon acid)–2 mM $MgSO_4$ at the indicated pH to a final protein concentration of 1.0 mg/ml. The membrane potential ($\Delta\Psi$; circles), pH gradient (ΔpH; squares), and lactate gradient ($\Delta\tilde{\mu}_{lac}/F$; triangles) were determined after 5 min of metabolism in the presence of 10 mM potassium-L-malate (open symbols) and 10 mM glucose (closed symbols). The total proton motive force of L-malate-fermenting cells is shown as a solid line.

For generation of metabolic energy by electrogenic lactate efflux (Fig. 1), the lactate gradient ($\Delta\tilde{\mu}_{lac}/F$) has to exceed the membrane potential ($\Delta\Psi$) plus two times the pH gradient ($Z\Delta$pH), i.e., $\Delta\tilde{\mu}_{lac}/F > \Delta\Psi - 2Z\Delta pH$ (10). The L-lactate gradient in L-malate-metabolizing cells, estimated from the distribution of L-[^{14}C]lactate, appeared to somewhat lower than the pH gradient (Fig. 2), arguing against electrogenic lactate efflux under these conditions.

Since the highest values of the proton motive force were reached at pH 5, these conditions were used to compare ATP synthesis by malolactic fermentation and glycolysis. In the presence of L-malate, L-lactate-loaded cells of *L. lactis* IL1403 rapidly synthesized ATP (Fig. 3). In comparison with glucose-metabolizing cells, the intracellular ATP concentration increased faster during malolactic fermentation but the final level reached was lower. ATP synthesis by malolactic fermentation was completely inhibited by nigericin in the presence and absence of valinomycin. Notice that at pH 5.0 and in the presence of nigericin, the intracellular pH is approximately 4.5 (20). *N,N*-dicylclohexylcarbodiimide (DCCD) inhibited the rate of ATP synthesis significantly, but because of partial inhibition of F_0F_1-ATPase activity, ATP

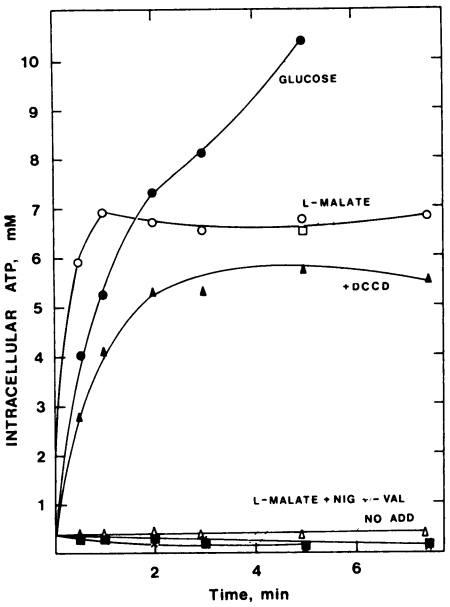

FIG. 3. Intracellular ATP concentrations. L-Lactate (100 mM)-loaded *L. lactis* IL1403 cells were diluted 100-fold into 50 mM K-MES plus 2 mM $MgSO_4$, pH 5.0, containing 20 mM potassium-L-malate or 10 mM glucose. At the times indicated, samples were withdrawn and analyzed for ATP content. Valinomycin (val) and nigericin (nig) concentrations were 2 and 1 μM, respectively. DCCD, cells treated with 100 μM DCCD for 1 h at 30°C; no add, no exogenous energy source present. The experiments were carried out at 30°C.

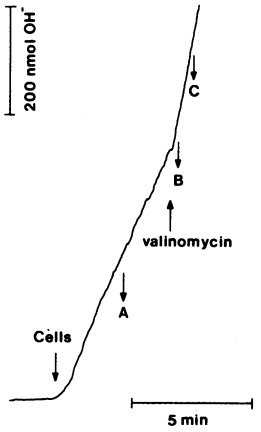

FIG. 4. Measurement of malolactic fermentation activity. At the times indicated, 10 μl of L-lactate (50 mM)-loaded *L. lactis* IL1403 cells (33.2 mg of protein per ml) were diluted into 4 ml of K-MES/KCl buffer, pH 5.0, containing 5 mM potassium-L-malate. The pH trace indicates the alkalinization of the medium. Arrows indicate the addition of cells and valinomycin (1 μM, final concentration); A, B, and C indicate when samples were withdrawn for the determination of L-lactate (see Table 1).

synthesis could not be completely abolished. The partial inhibition of ATPase activity was inferred from experiments in which the generation of a membrane potential by DCCD-treated and untreated cells metabolizing glucose (or lactose) was compared. Under these conditions, generation of the membrane potential is dependent on the activity of the F_0F_1-ATPase. DCCD-treated cells still generated a membrane potential, albeit with a rate that was at least 10 times lower than that of control cells; the steady-state value reached was approximately 50% that of untreated cells (data not shown). ATP synthesis by malolactic fermentation at pH 7 was much lower than at pH 5; i.e., the intracellular ATP concentration increased to values only three to four times those of resting cells (not shown). Altogether, these results indicate that malolactic fermentation generates a proton motive force that drives the synthesis of ATP via the F_0F_1-ATPase.

Malolactic fermentation. Malolactic fermentation involves the decarboxylation of L-malate to L-lactate and carbon dioxide. Since malic acid has one more carboxylic group than does lactic acid, alkalinization of the medium can, in principle, be used to estimate the rate of metabolism (Fig. 4). To validate the use of a pH electrode to measure malolactic fermentation activity, the proton consumption was compared with the production of L-lactate. As shown in Table 1, the H^+/L-lactate stoichiometry was always found to be close to 1. In accordance with the models proposed for metabolic energy generation by malolactic fermentation (Fig. 1), dissipation of the membrane potential by valinomycin resulted in a stimulation of the activity (Fig. 4). Stimulation by valinomycin was observed irrespective of the presence of nigericin, indicating that the stimulation cannot be explained by an increase in pH gradient only. At pH 5, the affinity constant of malolactic fermentation for L-malate was 4.3 mM; the maximal rate of fermentation was 1.96 μmol/min/mg of protein. The rate of malolactic fermentation at pH 6.5 with 10 mM L-malate as the substrate was 0.3 μmol/min/mg of protein (data not shown).

Resting (washed) cells of *L. lactis* IL1403 displayed a lag phase for malolactic fermentation (Fig. 5A, unloaded). To discriminate between the models proposed for malate uptake (Fig. 1), the effect of preloading of the cells with L-lactate on the initiation of malolactic fermentation was tested. When the cells were loaded with 50 mM L-lactate, malolactic fermentation started almost immediately (Fig. 5A). The maximal effect of preloading with L-lactate was observed with initial intracellular concentrations of 50 to 100 mM (not shown). Stimulation of the initiation of malolactic fermentation was specific for L-lactate (and to a lesser extent for D-lactate) and was not due to the generation of a pH gradient caused by a lactate diffusion potential, since preloading with acetate, benzoate (Fig. 5A), or bicarbonate (not shown) had little or no effect. Furthermore, dissipation of the pH gradient by nigericin did not nullify the initial stimulation of malolactic fermentation by loading of the cells with L-lactate; in fact, the small lag phase observed with L-lactate-loaded cells disappeared completely in the presence of nigericin (Fig. 5A, broken line). The apparent lag phase in

TABLE 1. Stoichiometry of malolactic fermentation[a]

Sample	H^+ consumption (μmol/mg)	Lactate production (μmol/mg)	H^+/lactate stoichiometry
A	4.7	4.3	1.08
B	9.0	8.6	1.04
C	11.6	11.8	0.98

[a] Samples correspond to those indicated in Fig. 4. At various times, 0.5-ml samples were removed from the electrode vessel, and the supernatant obtained after centrifugation was analyzed for lactate by gas chromatography. H^+ consumption was estimated from the pH traces directly. For details, see the legend to Fig. 4.

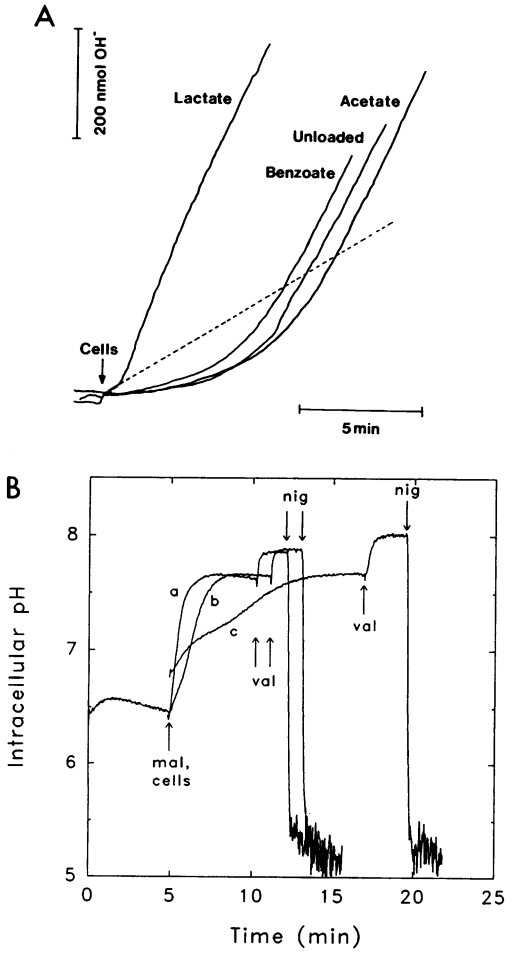

FIG. 5. Effect of intracellular L-lactate on the initiation of malolactic fermentation by *L. lactis* IL1403. (A) Measurement of malolactic fermentation activity. Reaction conditions were identical to those described for Fig. 4 except that cells were unloaded or loaded with 50 mM of L-lactate, acetate, or benzoate. The broken line represents malolactic fermentation of L-lactate-loaded cells in the presence of nigericin (0.5 μM, final concentration). (B) Changes in the intracellular pH during the initiation of malolactic fermentation. The intracellular pH was estimated from the changes in BCECF fluorescence as described in Materials and Methods. Trace a, L-lactate-loaded (50 mM) cells were diluted 300-fold into K-MES buffer, pH 5.0 (at t = 5), containing 10 mM potassium-L-malate; trace b, L-lactate-loaded cells were diluted into K-MES buffer without L-malate (at t = 0), and after 5 min of incubation, L-malate was added to 10 mM, final concentration; trace c, unloaded cells were diluted into K-MES buffer containing 10 mM L-malate (at t = 5). Valinomycin (val) and nigericin (nig) were added to final concentrations of 2.7 and 1.3 μM, respectively.

the absence of nigericin is due to the relative impermeability of the cell membrane for protons (and hydroxyl ions), which results in a delay in the appearance of the hydroxyl ions externally. The decreased rate of malolactic fermentation in the presence of nigericin is most likely due to an effect of the intracellular pH on the activity of malolactic enzyme (activity is reduced at low pH [19]).

To monitor the initial changes in the intracellular pH by malolactic fermentation, *L. lactis* IL1403 cells were loaded with the fluorescent pH indicator probe BCECF (14). Figure 5B shows a rapid initial alkalinization of the cytoplasm with L-lactate-loaded cells (trace a). If L-malate is added 5 min after dilution of L-lactate-loaded cells into L-lactate-free medium (trace b), the rate of increase of the intracellular pH is reduced, most likely because of a decreased internal concentration of L-lactate. For electroneutral efflux of lactate (or passive diffusion of lactic acid), the internal L-lactate concentration will decrease until equilibrium between the L-lactate and pH gradient is reached, i.e., at an intracellular concentration of approximately 10 mM (external lactate concentration is 330 μM; pH gradient is 1.5). The rapid initial phase of alkalinization is not observed with unloaded cells (trace c). Consistent with the uncoupling action of weak organic acids, resting cells maintained a higher intracellular pH in the absence than in the presence of L-lactate. The intracellular pHs estimated from this experiment (Fig. 5B) are somewhat higher than those of Fig. 2 because of the higher temperature (30 versus 20°C) at which the cells were incubated with L-malate.

In conclusion, the effects of loading of cells with L-lactate support the hypothesis that in vivo L-malate is transported in exchange for L-lactate. The electrogenicity of the antiport, i.e., L-malateH^-/lactic acid or L-malate^{2-}/L-lactate$^-$ antiport, can be inferred from the stimulation of fermentation by valinomycin (Fig. 4). To substantiate further the mechanism by which L-malate is taken up, transport experiments were performed with isolated membranes.

Transport of L-malate and L-lactate. Since L-malate taken up by the cells is rapidly converted into L-lactate and carbon dioxide, it is not possible to perform transport studies under these conditions. Initial experiments with membrane vesicles derived from *L. lactis* IL1403 indicated that some malolactic enzyme remained associated with the membrane preparations. Therefore, mutants defective in malolactic fermentation were isolated and characterized with respect malolactic enzyme and transport activity (see Materials and Methods). One of the mutants (IL1441-SO1) devoid of malolactic enzyme activity and exhibiting high transport activity was used for further studies. For practical reasons, membranes are more permeable for L-lactate (L-lactic acid) than for L-malate (L-malic acid); several transport reactions (Fig. 6 and 7) were assayed in the direction opposite the in vivo situation.

To demonstrate L-malate/L-lactate and L-malate/L-malate antiport, vesicles were loaded with L-[^{14}C]malate and diluted into media with various concentrations of L-lactate or L-malate or without further additions (Fig. 6). A rapid exit of L-[^{14}C]malate was observed in the presence of L-lactate (Fig. 6A) and L-malate (Fig. 6B) externally, demonstrating heterologous and homologous exchange by the transport system. At pH 5.9 and with 5 mM L-malate internally, the apparent affinity constants (K_Ts) for L-lactate and L-malate at the outer surface of the membrane are approximately 1 and 0.5 mM, respectively. Slow but significant release of L-[^{14}C]malate was observed in the absence of a countersubstrate (efflux). Although the efflux of L-[^{14}C]malate could be due to passive diffusion (pK_a^1 and pK_a^2 of L-malate are 3.4 and 5.2, respectively; the experimental pH was 5.9), it is very possible that the transport system also catalyzes transport of L-malate without countertransport of L-lactate. In fact, membrane vesicles of *L. lactis* ML3 (deficient in malolactic fermentation) exhibited negligible efflux under the same conditions, suggesting that the efflux observed with vesicles from *L. lactis* IL1441-SO1 is most likely carrier mediated.

The models for malate/lactate antiport and malate uptake presented in Fig. 1 indicate that both reactions are electrogenic; i.e., a membrane potential, inside negative, inhibits

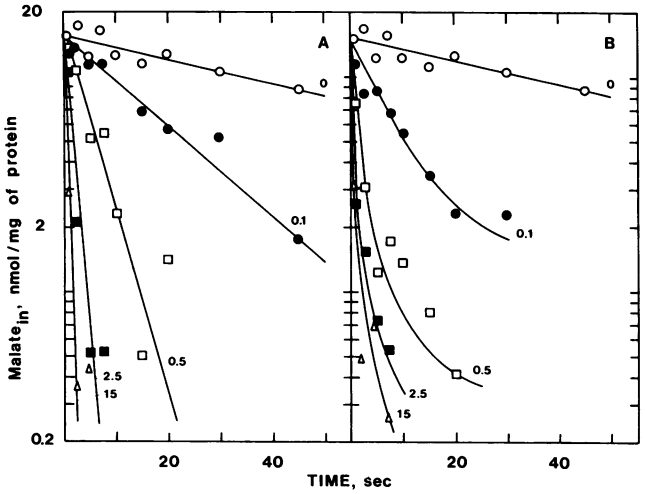

FIG. 6. Efflux and exchange of L-malate in membrane vesicles of *L. lactis* IL1441-SO1. Membrane vesicles were loaded with 5 mM potassium-L-[^{14}C]malate in KPi/KCl buffer, pH 5.9, as described in Materials and Methods. Efflux (open circles) and exchange (other symbols) were assayed at 25°C upon 80-fold dilution of the membrane vesicles into KPi/KCl buffer containing various concentrations of potassium-L-lactate (A) and potassium-L-malate (B) (concentrations [millimolar] are indicated). The final protein concentration in the assay mixture was 0.39 mg/ml.

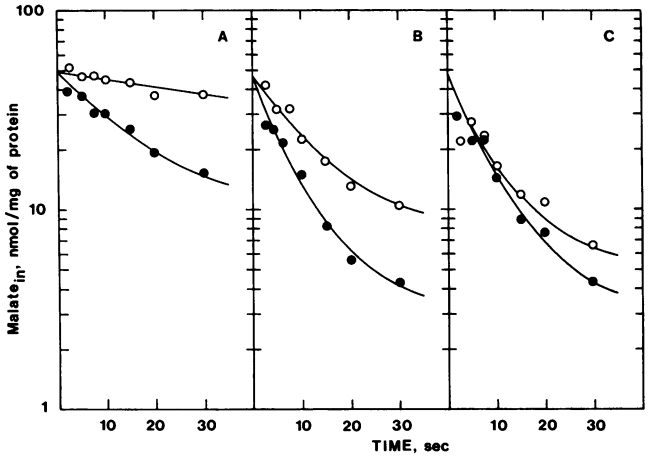

FIG. 7. Effect of membrane potential on efflux and exchange of L-malate in membrane vesicles of *L. lactis* IL1441-SO1 fused with liposomes. Fused membranes were loaded with 6.5 mM potassium-L-[^{14}C]malate in KPi/KCl buffer, pH 5.0, in the presence of valinomycin (2 nmol/mg of protein) as described in Materials and Methods. L-[^{14}C]malate efflux (A), heterologous L-[^{14}C]malate/L-lactate exchange (B), and homologous L-[^{14}C]malate/L-malate exchange (C) were assayed at 25°C upon 80-fold dilution of the membranes into KPi/KCl buffer, pH 5.0 (open circles), or 50 mM sodium phosphate–100 mM NaCl–2 mM $MgSO_4$, pH 5.0 (closed circles). L-Lactate (B) and L-malate (C) were present as potassium (open circles) or sodium (closed circles) salts at final concentrations of 5 mM. The final protein concentration in the assay mixture was 0.15 mg/ml.

the uptake of L-malate. To demonstrate the electrogenicity of the transport, L-malate efflux and heterologous L-malate/L-lactate and homologous L-malate/L-malate exchange were analyzed in the presence and absence of a membrane potential. To generate a membrane potential, inside negative, potassium-loaded fused membranes in the presence of valinomycin were diluted into sodium-containing buffers (Fig. 7, closed circles). L-Malate efflux (Fig. 7A) and heterologous L-malate/L-lactate exchange (Fig. 7B) were stimulated by the membrane potential, whereas homologous L-malate/L-malate exchange (Fig. 7C) was not.

To study the transport of L-malate further, membrane vesicles derived from *L. lactis* IL1441-SO1 were fused with liposomes in which beef heart cytochrome *c* oxidase was incorporated. In these hybrid membranes, a membrane potential and pH gradient can be generated in the presence of the electron donor system ascorbate–TMPD–cytochrome *c*. In principle, the proton motive force generated can drive the uptake of solutes via secondary transport systems, resulting in accumulation of solutes into the vesicular interior. In the presence of the electron donor system, however, little or no accumulation of L-malate was observed (Fig. 8A, no add).

Depending on the L-malate species transported and/or the number of protons symported with L-malate, the components of the proton motive force may drive, not affect or counteract, the uptake of L-malate. If L-malate is transported in the dianionic form (without protons), the membrane potential (inside negative) will prevent uptake of L-malate, whereas the pH gradient will have no effect. If monoanionic L-malate (or dianionic L-malate in symport with a proton) is transported, the uptake will be driven by the pH gradient but counteracted by the membrane potential. Transport of L-malic acid (or malate plus two protons) will be driven by the pH gradient, and the membrane potential will have no effect. Only if dianionic L-malate is transported with three or more protons will both the membrane potential and the pH gradient drive the uptake. To discriminate between the four possibilities, the effects of the ionophores valinomycin and nigericin on the uptake of L-malate were studied. In the presence of valinomycin, the membrane potential is dissipated whereas the pH gradient is somewhat elevated (5). Under these conditions, accumulation of L-malate was observed at both pH 5.0 and pH 5.9 (Fig. 8A). In the presence of nigericin with or without valinomycin, L-malate was not significantly accumulated. These results strongly suggest that L-malate can be taken up with one proton (or transport of monoanionic L-malate; MH^- in Fig. 1), depicted as electrogenic malate uptake in Fig. 1. If transport of L-malate is electroneutral, significant accumulation in the absence of valinomycin is expected (see uptake of L-lactate).

Finally, attempts were made to characterize transport of L-lactate. In membrane vesicles of *L. lactis* IL1441-SO1, efflux of L-lactate was too fast to be analyzed accurately, most likely because of exit of lactic acid by passive diffusion. In membrane vesicles fused with cytochrome *c* oxidase-containing proteoliposomes, rapid uptake of L-lactate was observed in the presence of the electron donor system (Fig. 8B, no add). The accumulation of L-lactate was stimulated by valinomycin and totally abolished by nigericin (Fig. 8B). These results are consistent with electroneutral carrier-mediated transport and/or passive diffusion of L-lactic acid. The data contradict the proposal of Cox and Henick-Kling (2), which states that electrogenic lactate efflux (see Fig. 1) generates metabolic energy during malolactic fermentation.

DISCUSSION

We have demonstrated that malolactic fermentation results in the generation of a high proton motive force, which

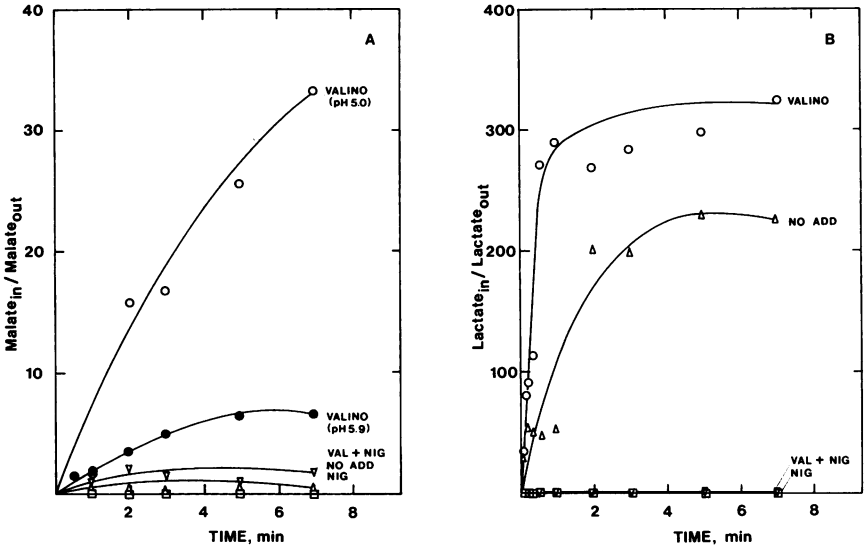

FIG. 8. Uptake of L-malate (A) and L-lactate (B) in membrane vesicles of *L. lactis* IL1441-SO1 fused with cytochrome *c* oxidase-containing liposomes. L-[^{14}C]malate (20 μM, final concentration) and L-[^{14}C]lactate (2.8 μM, final concentration) uptake by the fused membranes was assayed in KPi/KCl buffer, pH 5.0 (open symbols) and pH 5.9 (closed symbols), at 25°C and at final protein concentrations of 0.23 and 0.11 mg/ml for L-malate and L-lactate uptake, respectively. The electron donor system, potassium ascorbate–TMPD–cytochrome *c*, was present in all samples. Valinomycin (valino, val) and nigericin (nig) were added to final concentrations of 0.6 and 0.3 μM, respectively. No add, no addition.

in turn can drive ATP synthesis by the F_0F_1-ATPase. For the mechanism by which the proton motive force is generated, three distinct mechanisms of transport of L-malate and L-lactate in combination with proton consumption by decarboxylation have been considered (Fig. 1). Each mechanism generates the same amount of metabolic energy, i.e., the equivalent of one proton translocated per L-malate metabolized (or one-third ATP equivalent per turnover, given a stoichiometry of three H^+ per ATP for ATP synthesis by the F_0F_1-ATPase). On basis of the transport experiments, we conclude that transport of L-malate and L-lactate can occur as malateH^-/lactic acid (or malate^{2-}/lactate$^-$) antiport and malateH^- uniport (or malate^{2-}/H^+ symport) accompanied by carrier-mediated or passive efflux of lactic acid, which in both cases leads to the generation of a membrane potential. Evidence that electrogenic malate/lactate antiport is indeed occurring in vivo comes from experiments in which L-lactate and unloaded cells are compared with respect to the initiation of malolactic fermentation (Fig. 5). The question whether L-malate is taken up predominantly by the antiport reaction during malolactic fermentation cannot unequivocally be answered. The driving force for the uptake of L-malate by the antiport mechanism is supplied by the electrochemical gradients for L-malate plus L-lactate, whereas for the uniport mechanism the driving force is composed of the electrochemical gradients for L-malate and protons. Since the pH gradient in *L. lactis* IL1403 cells metabolizing L-malate is somewhat higher than the gradient for L-lactate (Fig. 2), the driving force for malate uptake by malateH^- uniport (or malate^{2-}/H^+ symport) is higher than that by malate/lactate antiport (note that only the driving force on the carrier is considered; depending on pH and species [L-malateH^- or L-malate^{2-}] transported, the effective accumulation may differ due to protonation or deprotonation of the solute). Despite a thermodynamic advantage, there may be kinetic reasons for the cell to favor the antiport reaction. Following binding of L-malate to the carrier at the outer surface of the membrane, transmembrane translocation takes place, after which L-malate is released into the cytoplasm. At this point, the unloaded carrier has to reorient binding site(s) before a second L-malate molecule can bind. By analogy with other carrier proteins (9, 10, 13, 18), the reorientation of the binding site(s) to the outer surface of the membrane may be faster when a solute, e.g., L-lactate, is bound. In this scheme, the contribution of the antiport reaction to the accumulation of L-malate would be more important than the uniport (or H^+ symport) of L-malate. In fact, the first-order rate constants for L-malate efflux in membrane vesicles are 1 to 2 orders of magnitude lower than for the antiport reactions (Fig. 6).

By catalyzing an antiport and a uniport (or H^+ symport) reaction, malate metabolism can be initiated, albeit slowly (Fig. 5), in the absence of intracellular lactate. This situation clearly differs from that of arginine metabolism in *L. lactis*, in which case a second carrier protein is required for funneling a countersubstrate of the arginine/ornithine antiporter into the cell (6, 7, 18).

The electrogenic malate/lactate antiport of *L. lactis* described in this report resembles the oxalate/formate antiport of *O. formigenes* (1). One could speculate that the antiport system of *O. formigenes* also catalyzes uniport (or H^+ symport, depending on the species transported); however, in contrast to L-malate-loaded membranes of *L. lactis* (Fig. 6 and 7), oxalate-loaded proteoliposomes of *O. formigenes* exhibit little or no efflux activity (see Fig. 4 in reference 1).

L-Malate/L-lactate antiport and L-malateH^- uniport (or L-malate^{2-}/H^+ symport) could be catalyzed by separate transport proteins. However, the isolation of a malolactic fermentation-negative mutant defective in transport of L-malate (UV1 [23]) does not support this idea.

Although L-lactate can leave the cell via the antiport reaction, the lipophilic nature of the molecule will also permit it to diffuse out passively. The production of L-lactic acid intracellularly and the pH gradient, inside alkaline, will

result in accumulation of L-lactate, and as a consequence the antiport reaction may not run short of L-lactate. The carbon dioxide (or bicarbonate) produced by malolactic fermentation does not appear to be a substrate for the antiport reaction.

The transport mechanisms for L-malate (and L-lactate), i.e., electrogenic malate/lactate antiport and electrogenic malate uptake, impose a strong pH dependence on the metabolic energy that can be derived from malolactic fermentation. For both transport mechanisms, the membrane potential inhibits uptake of L-malate, whereas the pH gradient stimulates directly (electrogenic malate uptake) or indirectly (by affecting the L-lactate gradient; electrogenic malate/lactate antiport). At high pH the membrane potential generated by malolactic fermentation (and glycolysis) is high compared with the pH gradient, whereas at low pH the pH gradient and membrane potential are similar or the pH gradient may exceed the membrane potential (Fig. 2) (21). Consequently, at high pH, uptake of L-malate, and consequently malolactic fermentation, is slow, leading to reduced synthesis of ATP in comparison with uptake at low pH (see Results). Data on the mechanism of transport of L-malate and L-lactate indicate that malolactic fermentation will result in significant increases in growth yield at low pH values only.

ACKNOWLEDGMENT

The research of B. Poolman has been made possible by a fellowship from the Royal Netherlands Academy of Arts and Sciences.

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